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# Characterizations of Novel Poly(aspartic acid) Derivatives Conjugated with γ-Amino Butyric Acid (GABA) as the Bioactive Molecule

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Novel poly(aspartic acid) derivatives conjugated with γ-amino butyric acid, GABA, moieties, and their amphiphilic analogs were synthesized and characterized. The chemical structures of these polymers were confirmed by FT-IR and $^1$H NMR spectroscopy. Their physicochemical properties in aqueous media were characterized by electrophonetic light scattering spectrophotometry (ELS), acid-base titration, and UV-spectroscopy. In addition, the *in vitro* cell activity of the GABA-conjugated polymer was examined. These results indicated that GABA-conjugated poly(aspartic acid) derivatives showed cell-growth activity and nanoparticle formation of a suitable size within aqueous media. These polymers have potential application in the cosmetic and pharmaceutical fields.

**Key Words**: Poly(aspartic acid), Bioconjugation, γ-Amino butyric acid (GABA), Nanoparticle, Cell-growth activity

## Introduction

The importance of polymeric materials incorporating biodegradability and biocompatibility to various biomedical applications is being recognized. Thus, the macromolecular design and synthesis of these polymers have been extensively studied in recent years. Poly(amino acid), which has a protein-like amide linkage, is known to be biodegradable and is thus used as medical, cosmetic, fabric, and metal absorbent materials.[1] Poly(aspartic acid) (PASP), a poly(amino acid), is a promising water-soluble and biodegradable polymer, which is commonly obtained from the hydrolysis of polysuccinimide (PSI).[2,3] PSI is prepared by thermal polycondensation of an L-aspartic acid monomer.[4-6]

Studies on the amphiphilic poly(aspartic acid) derivatives have recently been reported by several research groups.[7-10] Amphiphilic graft copolymers have attracted considerable interest because of their various industrial applications and relatively simple preparation methods compared to block copolymers. Due to their amphiphilic characteristics, block and graft copolymers containing both hydrophobic and hydrophilic components can be used to stabilize dispersions and emulsions, as well as for surface modification, drug delivery carriers, and nano-reactors.

Amino acids are used in various fields including nutrition, pharmaceuticals, cosmetics, and agrochemicals. Amino acids can function as spacers or bioactive molecules. Amino acid-immobilized polymers and poly(amino acid)s are known to have specific pH sensitivities.[13-16] Also, the amino acids in immune responses can be used in developing effective strategies to improve health and prevent infectious diseases.[17] γ-Amino butyric acid, GABA, is an important non-essential amino acid and is known to play roles in various biological systems. Glutamic acid, GABA, and glycine are neurotransmitters that bind to specific receptors in the vertebrate nervous system and mediate synaptic transmission. Of these amino acids, GABA is the most widely distributed amino acid inhibitory neurotransmitter in the vertebrate central nervous system.[18]

The conjugation of biologically active molecules to surfaces and carrier systems is a vital technique in a variety of biomedical and cosmetic applications including targeted drug delivery and biosensing. Bioactive compounds can be natural or synthetic, and are defined as compounds which catalyze or elicit a specific response within a given biological system. Polymers are particularly important in bioconjugation due to their variety of properties accessible throughout a range of polymer families, including biocompatibility and stimuli-responsive properties. In the biomedical field, a covalent immobilization can be used to extend the half-life of a biomolecule, prevent its metabolism, or to allow continued bioactivity of in-dwelling devices.[19] Carbodiimides are most commonly used as coupling reagents to obtain an amide linkage between a carboxylate and an amine, or a phosphoramidate linkage between a phosphate and an amine.[20]

Novel biodegradable graft copolymers based on poly(aspartic acid) containing pendent GABA moieties were synthesized in this work. A GABA component conjugated to the polymer backbone can provide pH-sensitivity and a specific biological function *in vivo*. The chemical structures of the polymers were confirmed by FT-IR and $^1$H NMR spectroscopy. The physicochemical properties in aqueous media were characterized by an electrophonetic light scattering spectrophotometer (ELS) measurement, acid-base titration, and field emission scanning electron microscopy (FE-SEM). In addition, a preliminary test on their cell activities was conducted using the HDF-N human cell line.

## Experimental

**Materials and Instruments.** L-aspartic acid (98+%), *o*-phosphoric acid (98%), γ-amino butyric acid (GABA, 99%), 1-hexadecylamine (98%, HDA), *N*,*N*-dimethylformamide (99.8% anhydrous, DMF), dimethyl sulfoxide (99.9+% ACS reagent,

DMSO), and *N*,*N*′-dicyclohexyl carbodiimide (99%, DCC) were purchased from Aldrich Chemical Co. All other chemicals were of high quality and used without further purification. A dialysis membrane (Spectra/pore4 with MWCO 3500 and 12000-14000) was used to eliminate any unreacted monomers and solvent. $^1$H-NMR spectra were recorded with a Bruker AMX-500 spectrometer (Karlsruhe, Germany). The FT-IR spectra were obtained with a Bruker Tensor27. The size and distribution of self-aggregates (nanoparticles) were measured by ELS-Z2 (ELS-8000, Otsuka Electronics, Japan). Other physicochemical properties of the polymers were confirmed by acid-base titration and UV-spectrophotometry (Biochrom Libra S22, Cambridge, UK). The nanoparticle morphology was observed by field emission scanning electron microscopy (FE-SEM, JSM6700F, JEOL, Japan).

**Measurements.** The particle size distribution in aqueous solution (0.1 wt%) was determined using an ELS-Z2 (ELS-8000, Otsuka Electronics, Japan) with a laser light wavelength of 638 nm and a scattering angle of 165°. The polymer powder was dispersed magnetically in aqueous solution for 24 h and then filtered using a 0.45 μm syringe filter disc to remove oversized material before measurement. The turbidity of the polymer solution at different pH value was determined by UV-spectrophotometer at 500 nm and the polymer concentration was 1 wt%. An acid-base titration of the GABA-conjugated copolymer was conducted as follows: the 1 wt% polymer solution was titrated to pH 11 with 1 N NaOH, and then the pH of the solution was adjusted by incrementally adding a 0.1 N HCl solution.

The cell growth activity of the poly(aspartic acid) derivatives were determined with a cell growth test using the HDF-N human cell line (P1C, ELISA). First, the HDF-N was incubated in a 75-T flask at 37 °C, with 5% $CO_2$ using FGM-2 (fibroblast growth media, clonetics). The culture solution was removed and washed with PBS. The cells were separated from the FGM-2 by inserting 0.5 mL of a Trypsin-EDTA solution. The dispersed cells were then centrifuged at 1100 rpm for 5 min. Second, for the cell culture, $2.0 \times 10^4$ cells/mL of HDF-N human cells were used in the FGM-2 growth media and in the 135 μL/well DMEM (FBS 0.2%, BSA 0.1%) test media. After 24 h incubation, a 15 μL of poly(aspartic acid) derivative solutions in water (0.0001 ~ 0.1%) were added. After an additional three days of incubation, the NRU test was carried out (P1C, ELISA).

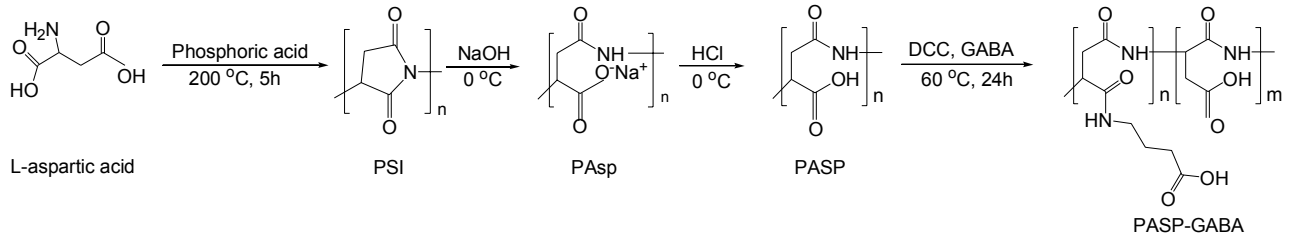
**Synthesis of GABA-Conjugated Poly(aspartic acid), PASP-GABA.** The synthesis of poly(aspartic acid), PASP, is produced from the hydrolysis of polysuccinimide (PSI), the thermal condensation polymer of L-aspartic acid monomer as the procedure was described in our previous work.[21] PASP and DCC (1.5 mole equivalent of aspartic acid residue) were dissolved in DMF/distilled water in a three-neck flask. The solution was stirred for 3 h in a water bath at 60 °C, and then a molar equivalent of GABA was added. After stirring for 24 h, the solution was precipitated in 8-fold acetone and centrifuged. The recovered powdery product was dissolved in distilled water and dialyzed using a membrane (MWCO 12,000-14,000) to remove all unreacted low molecular weight impurities. Finally, the dialysis product was freeze-dried to obtain PASP-GABA (yield 53%). $^1$H-NMR (500 MHz, $D_2O$): δ 2.5-3.3 (m, 2H, CH-*CH2*-CO-NH), 4.5-4.7 (m, 1H, NH-*CH*-CO-CH2), 3.1-3.3 (c, 2H, NH-*CH2*-CH2-CH2-COOH), 1.6-1.9 (c, 2H, NH-CH2-*CH2*-CH2-COOH), 2.1-2.5 (c, 2H, NH-CH2-CH2-*CH2*-COOH)

**Synthesis of GABA-Conjugated PASP-HDA, PASP-HDA-GABA.** Polysuccinimide (PSI) and hexadecylamine (HDA) were dissolved in DMF in a three-neck flask. The solution was stirred for 7 h. After the reaction, the solution was precipitated into 8-fold methanol and the precipitate was filtered and washed with methanol, then dried under vacuum at 25 °C. The hexadecylamine-grafted PSI (PSI-HDA) prepared above was dispersed in distilled water. A 0.1 N Sodium hydroxide solution was slowly dropped into the PSI-HDA dispersion, while keeping the solution pH below 10.8 at room temperature. After the mixture was stirred overnight, 0.1 N HCl was added to the solution until the pH remained at 4.0 in an ice bath. Then, the solution was precipitated in 8-fold acetone and centrifuged. The powdery product was dissolved in distilled water and purified by dialysis using a membrane (MWCO 12,000-14,000). Finally, the dialyzed product was freeze-dried.

PASP-HDA and DCC (1.5 equivalent of aspartic acid residue) was dissolved in DMSO/distilled water in a three-neck flask. The solution was stirred for 3 h in a water bath at 50 °C, then GABA (1.0 equivalent of aspartic acid residue) was introduced. After the reaction, 100 mL of distilled water was added and the solution was stirred for 10 min then filtered. The filtrate was dialyzed (using membrane MWCO 3500) to remove the un-reacted monomer and residual solvent. After dialysis, the product was filtered again. Finally, the product was freeze-dried. $^1$H-NMR (500 MHz, $D_2O$): δ 2.7-2.95 (m, 2H, CH-*CH2*-CO-NH), 4.32-4.7 (m, 1H, NH-*CH*-CO-CH2), 3.1-3.3 (c, 2H, NH-*CH2*-CH2-CH2-COOH), 1.6-1.9 (c, 2H, NH-CH2-*CH2*-CH2-COOH), 2.1-2.5 (c, 2H, NH-CH2-CH2-*CH2*-COOH), 1.3-1.5 (g, 2H, NH-*CH2*-(CH2)14-CH3), 1.1-1.3 (g, 2H, NH-CH2-(*CH2*)14-CH3), 0.75-0.98 (g, 3H, NH-CH2-(CH2)14-*CH3*)

## Results and Discussion

**Synthesis and Characterization of GABA-Conjugated Poly (aspartic acid)s.** Poly(aspartic acid), PASP, is produced from the hydrolysis of polysuccinimide (PSI), the thermal condensation polymer of L-aspartic acid monomer.[21] The molecular weight of PSI was measured to be Mn of 136,000 g/mol (PDI 1.17) by gel permeation chromatography using polystyrene standards and DMF as the eluent. γ-Amino butyric acid, GABA, was conjugated to poly(aspartic acid) using DCC as the coupling reagent (Scheme 1). The FT-IR spectra of (A) PSI, (B) PASP, and (C) PASP-GABA are shown in Figure 1. Spectrum (A) shows the characteristic absorption bands of an imide ring at 1727 $cm^{-1}$ and 1393 $cm^{-1}$. Spectrum (B) shows the characteristic absorption bands of an amide at 1610 $cm^{-1}$ and 1530 $cm^{-1}$, as well as a broad band around 3380 $cm^{-1}$ corresponding to an OH group. In Spectrum (C), the absorption bands at 1500-1700 $cm^{-1}$ shifted with the introduction of an amino acid moiety to the pendants. The PASP-GABA structure was also confirmed by $^1$H-NMR measurement. Figure 2 shows the $^1$H-NMR spectrum of PASP-GABA. The proton peaks **c**, **d**, and **e** were assigned to three different methylene protons of GABA as indicated on the structure. The DS (degree of substitution) of GABA was calculated by comparing the peak intensity of the methine




**Scheme 1.** Synthesis of GABA-conjugated PASP (PASP-GABA)

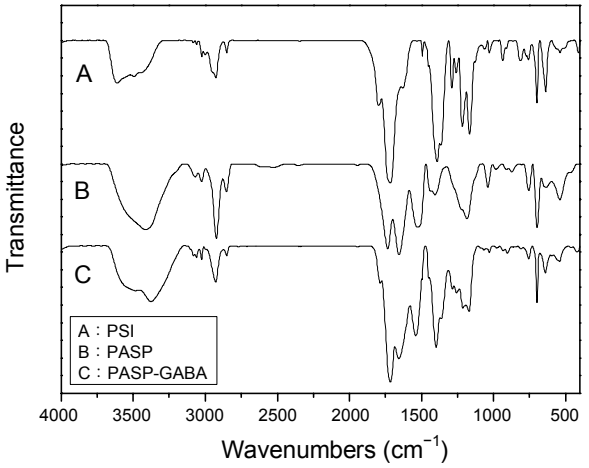



**Figure 1.** FT-IR spectra of PSI (A), PASP (B), and PASP-GABA (C).

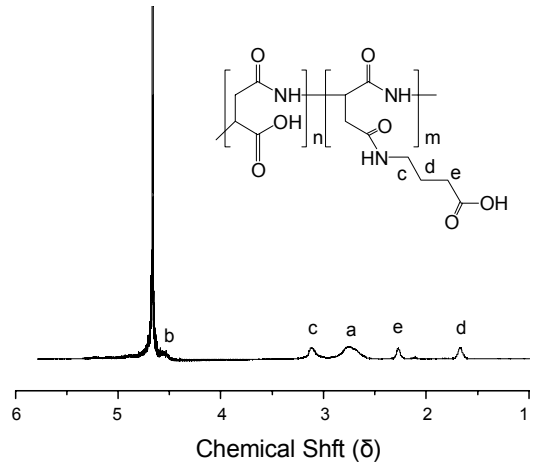
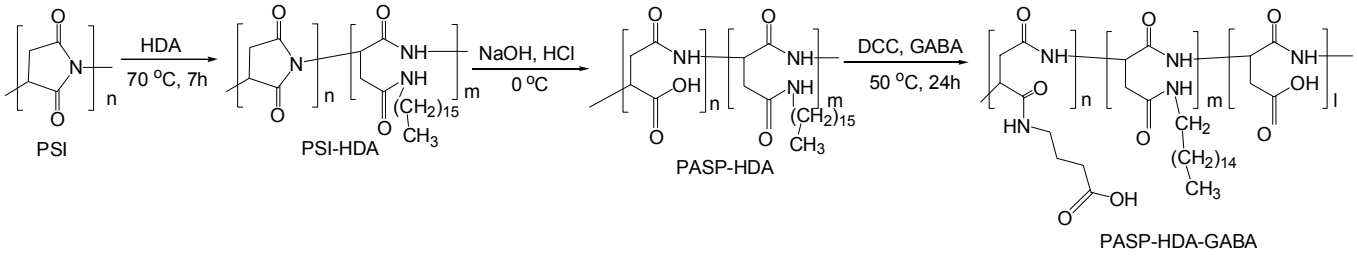



**Figure 2.** $^1$H-NMR spectrum of PASP-GABA ($D_2O$).




**Scheme 2.** Synthesis of GABA-conjugated amphiphilic polyaspartamides

proton of the polyaspartamide backbone at **b** with the methylene proton of the GABA moiety at **e**. The DS of GABA per aspartic acid unit was approximately 44%.

PASP-HDA-GABA, an amphiphilic poly(aspartic acid) derivative, was prepared with the reaction of PASP-HDA with GABA (Scheme 2). PASP-HDA was prepared from the aminolysis reaction of PSI with HDA and the following hydrolysis reaction. From the $^1$H NMR analysis, the HDA content was determined to be 23% per repeating unit. The carboxylic group of PASP-HDA was then coupled with the primary amine of GABA using DCC to obtain PASP-HDA-GABA.[22,23] Figure 3 shows the FT-IR spectra of (A) PSI-HDA, (B) PASP-HDA, and (C) PASP-HDA-GABA. Spectrum (A) shows the characteristic absorption bands of an imide ring at 1727 cm$^{-1}$ and 1393 cm$^{-1}$, as well as the absorption band of alkyl groups at 2950 cm$^{-1}$. Spectrum (B) shows the characteristic absorption bands of an amide group at both 1610 cm$^{-1}$ and 1530 cm$^{-1}$. Spectrum (C) shows multiple absorption bands of amide and carboxylate groups at 1500-1700 cm$^{-1}$, with a strong and broad absorption bands indicating hydroxyl and NH groups at 3200-3600 cm$^{-1}$. The PASP-HDA-GABA structure was also confirmed by $^1$H-NMR analysis as shown in Figure 4. The proton peaks of **f**, **g**, and **h** were assigned to the methylene protons of the GABA moiety, and the proton peaks of **c**, **d**, and **e** were assigned to the methylene and methyl protons of the HDA pendant. The DS of GABA was calculated by comparing the integral area of the methyl proton of HDA (**e**) with the methylene proton of GABA (**h**). The FT-IR and $^1$H-NMR analyses indicated that the PASP-HDA-GABA was successfully prepared.

**Physicochemical Properties of GABA-Conjugated Amphiphilic Polyaspartamides in Aqueous Media.** The compositions and physicochemical properties of typical amphiphilic PASP derivatives are summarized in Table 1. The particle size distributions of amphiphilic PASP-HDA and PASP-HDA-GABA (A and B) were measured by ELS. The average diameter of the particles in PASP-HDA was about 28 nm with a narrow distri-

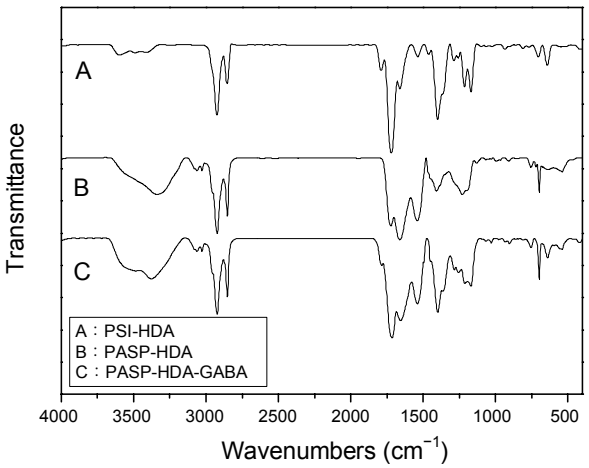



**Figure 3.** FT-IR spectra of PSI-HDA (A), PASP-HDA (B), PASP-HDA-GABA (C).

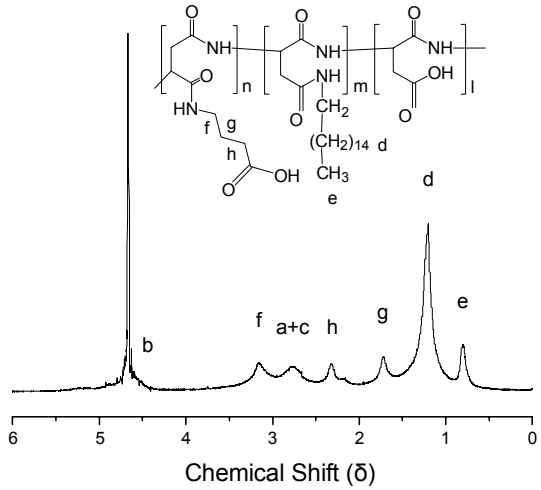



**Figure 4.** $^{1}$H-NMR spectrum of PASP-HDA-GABA ($D_2O$).

**Table 1.** Conditions and Results of Polymerization

| Sample | Solvent | Temp. (°C) | DS (HDA) | DS (GABA) | Yield (%) | Average Diameter (nm) |
|---|---|---|---|---|---|---|
| PASP-HDA | Water | 0 | 23% | 0% | 81 | 28 |
| PASP-HDA-GABA(A) | DMSO/Distilled Water (2/1) | 50 | 23% | 36% | 76 | 45 |
| PASP-HDA-GABA(B) | DMSO/Distilled Water (2/1) | 50 | 23% | 67% | 72 | 30 |

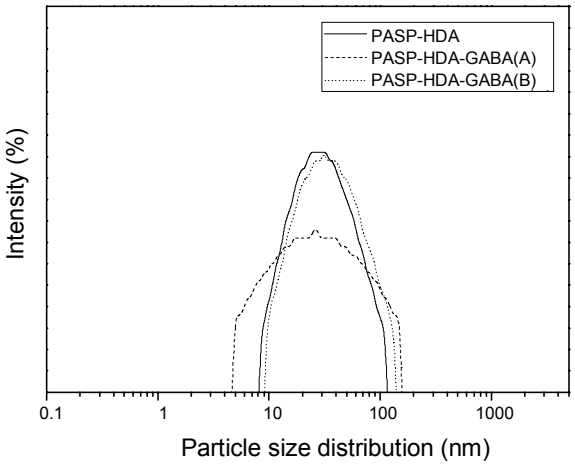



**Figure 5.** Particle size distributions of amphiphilic polyaspartamides in PBS (pH 7.4).

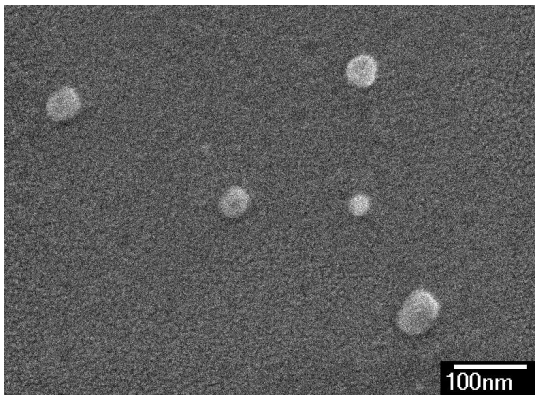



**Figure 6.** FE-SEM image of PASP-HDA-GABA nanoparticle.

bution. After GABA conjugation, the particle size distribution changed, and the average diameters of the nanoparticles slightly increased to 45 and 35 nm for the PASP-HDA-GABA (A) and (B), respectively (Figure 5). As shown in the figure, the PASP-HDA-GABA(A) possessed a rather broad distribution compared to the other two samples. With GABA conjugation and the content, the hydrophilic and hydrophobic balance changed to allow for the molecular reorganization of the polymers, resulting in a different aggregation state. A further examination on the composition-dependent changes of the particle size distribution is out of scope of this report. Figure 6 shows a typical FE-SEM image of this nanoparticle from amphiphilic PASP-HDA-GABA(B). The nanoparticle formation and reasonable particle size of these GABA-conjugated poly(aspartic acid)s suggest a potential application of this material in particle-mediated epidermal delivery (PMED). The turbidity changes in the aqueous solutions as a function of pH were determined by UV-spectrophotometry (Figure 7). At higher pHs (over pH 4), both PASP-HDA and PASP-HDA-GABA were clear and showed high light transmittance. At pHs below 4, however, the solution became turbid due to the protonation of carboxylic pendants (pKa of GABA was 4.2), resulting in a partial precipitation of polymers. Figure 8 shows the acid-base titration profiles of PASP-HDA, PASP-HDA-GABA(A), and PASP-HDA-GABA

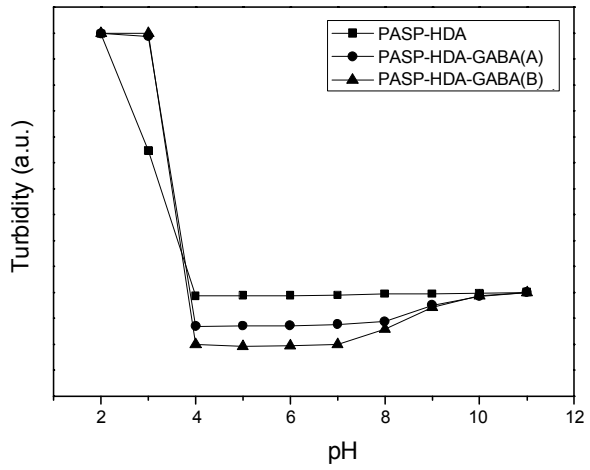



**Figure 7.** Turbidity change of amphiphilic polyaspartamides as function of solution pH.

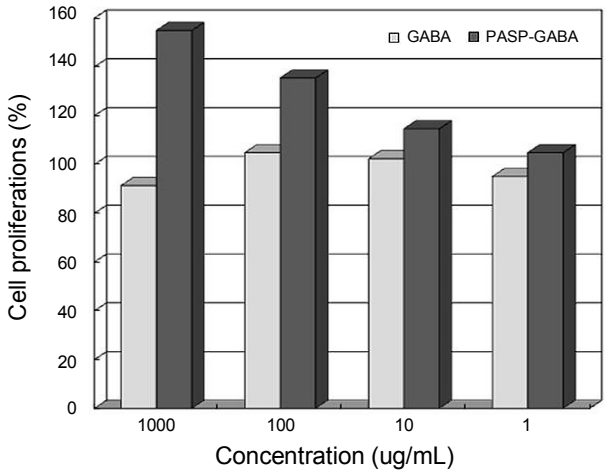



**Figure 9.** Cell growth activity of GABA-conjugated PASP.

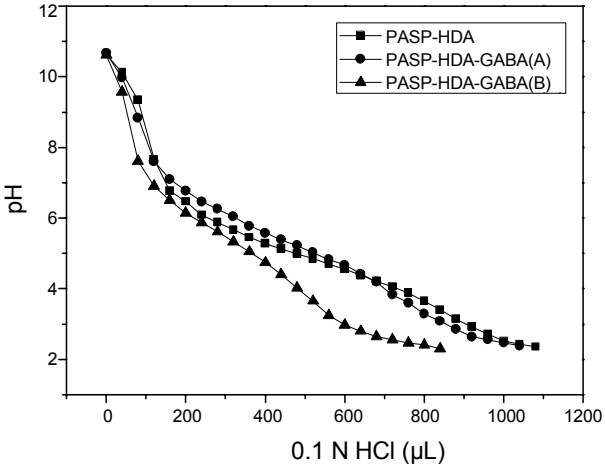



**Figure 8.** Acid-base titration profile of amphiphilic polyaspartamides.

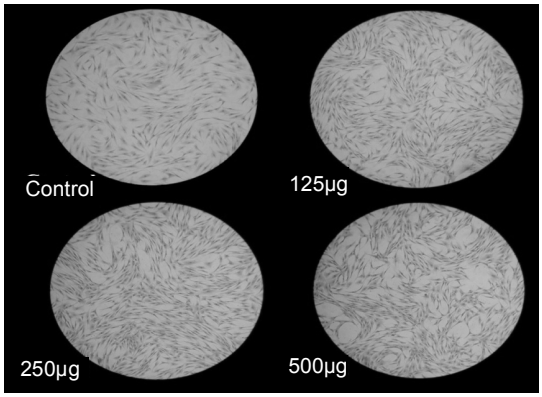



**Figure 10.** The microphotographs of cell growth at different concentrations of PASP-GABA.

(B), respectively. Starting from pH 11, which was obtained by adding NaOH, the aqueous solution was titrated with 0.1 N HCl in small increments. With the addition of 0.1 N HCl solution, the fully ionized carboxyl group in the polymer start to gain protons and the solution pH slowly decrease after reaching point at around pH 7. The pH of the polymer solutions decreased almost linearly. PASP-HDA-GABA(B), having a higher GABA content, showed a relatively rapid decrease compared to the other two polymers.

**Cell Activity of GABA-Conjugated Poly(aspartic acid) Derivatives.** The cell activity of GABA-conjugated poly(aspartic acid) was determined using the HDF-N human cell line (Figure 9). The results clearly demonstrated the cell growth activity of GABA and the polymer conjugate. As shown in the figure, the enhanced cell growth activity was observed when GABA-conjugated PASP was used as compared to those obtained by using GABA only. In addition the activity was improved linearly with increasing concentration. At a concentration of 100 μg/mL, the cell proliferation percentage of PASP- GABA was almost 40% higher than that of GABA alone. The microphotographs of HDF-N human cell growth at different concentrations of PASP-GABA are presented in Figure 10. These results indicated that PASP-GABA actively functioned in cell growth with the synergic effect of poly(aspartic acid) conjugation.

## Conclusions

γ-Amino butyric acid, GABA, was conjugated to poly(aspartic acid) using DCC as the condensing reagent, and also novel amphiphilic poly(aspartic acid) derivative with GABA pendants were synthesized and characterized. The GABA-conjugated poly(aspartic acid) exhibited enhanced cell-growth activity as evidenced by an NRU test using an HDF-N human cell line (P1C, ELISA). The particle formation and particle size distribution of the GABA-conjugated amphiphilic poly(aspartic acid)s were observed by FE-SEM and ELS, which showed spherical nanoparticles with the size in the range of 30 - 45 nm. These biocompatible, GABA-conjugated polymers have potential applications in the cosmetic and pharmaceutical fields.

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